

## DESCRIPTION

DIMERIZED PEPTIDE

## 5 TECHNICAL FIELD

The present invention relates to cancer vaccine therapy, more particularly to a peptide dimer which can produce a tumor antigen peptide having activity of inducing cytotoxic T cells, and a pharmaceutical composition comprising the same.

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## BACKGROUND ART

The cell mediated immunity, particularly a cytotoxic T cell (hereinafter, referred to as "CTL") plays a significant role in the in vivo rejection of tumor cells or virus-infected cells. CTLs recognize a complex  
15 between an antigen peptide ("tumor antigen peptide") derived from a tumor antigen protein and an MHC (major histocompatibility complex) class I antigen, which is referred to as "HLA antigen" in the case of human, on a cancer cell, and attack and kill the cell.

Typical examples of tumor antigen proteins include those listed in  
20 the Table of Immunity, vol.10:281, 1999. Specific examples include the melanosome antigens such as melanocyte tissue-specific protein gp 100 (J. Exp. Med., 179: 1005, 1994), MART-1 (Proc. Natl. Acad. Sci. USA, 91:3515, 1994) and tyrosinase (J. Exp. Med., 178: 489, 1993), and tumor markers as antigen proteins other than melanoma such as HER2/neu (J. Exp. Med.,  
25 181: 2109, 1995), CEA (J. Natl. Cancer. Inst., 87:982, 1995) and PSA (J. Natl. Cancer. Inst., 89:293, 1997).

A tumor antigen peptide is a peptide of around 8 to 11 amino acids that can be produced by intracellular processing of a tumor antigen protein by a protease in cells (Cur. Opin, Immunol., 5: 709, 1993 ; Cur. Opin,  
30 Immunol., 5: 719, 1993 ; Cell, 82: 13, 1995 ; Immunol. Rev., 146: 167,

1995). As described above, the so produced tumor antigen peptide is presented on the surface of a cell as a complex with an MHC class I antigen (HLA antigen) and recognized by CTLs. Accordingly, for the purpose of developing an immunotherapeutic agent for cancer (cancer vaccine) that makes use of the tumor cell destruction by CTLs, it is highly important to identify a tumor antigen peptide in a tumor antigen protein, which peptide is able to induce CTLs efficiently.

#### DISCLOSURE OF INVENTION

One of purposes of the present invention is to provide a novel tumor antigen derived from a tumor antigen peptide useful in vivo.

The present inventors have found that some peptides having been demonstrated to be a tumor antigen peptide contain a cysteine residue(s) and that a dimer composed of such peptides surprisingly show an activity of inducing CTLs ("CTL-inducing activity") equivalent to the monomer upon administration, and established the present invention.

Thus, the present invention encompasses the followings.

(1) A peptide dimer wherein two peptide monomers each consisting of 7-30 amino acids including at least one cysteine residue and being capable of producing a tumor antigen peptide having CTL-inducing activity are bound each other through a disulfide bond(s).

(2) The peptide dimer according to (1) above, which can produce a tumor antigen peptide having a CTL-inducing activity.

(3) The peptide dimer according to (1) or (2) above, wherein two peptide monomers are bound through one or two disulfide bonds.

(4) The peptide dimer according to any one of (1) to (3) above, wherein the peptide monomers are derived from WT1 that is an expression product of tumor suppressor gene.

(5) The peptide dimer according to any one of (1) to (4) above, wherein the peptide monomer is as follows:

Cys Xaa Thr Trp Asn Gln Met Asn Xaa (SEQ ID NO: 72)

wherein Xaa at position 2 is an amino acid residue selected from Tyr, Phe, Met and Trp; and Xaa at position 9 is an amino acid residue selected from Phe, Leu, Ile, Trp and Met.

- 5 (6) The peptide dimer according to any one of (1) to (4) above, wherein the peptide monomer is selected from the following peptides.

Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 11)

Asp Phe Lys Asp Cys Glu Arg Arg Phe (SEQ ID NO: 18)

Ala Tyr Pro Gly Cys Asn Lys Arg Tyr (SEQ ID NO: 19)

10 Asn Ala Pro Tyr Leu Pro Ser Cys Leu (SEQ ID NO: 20)

Gly Cys Asn Lys Arg Tyr Phe Lys Leu (SEQ ID NO: 21)

Arg Trp Pro Ser Cys Gln Lys Lys Phe (SEQ ID NO: 22)

Asp Ser Cys Thr Gly Ser Gln Ala Leu (SEQ ID NO: 23)

Cys Tyr Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 44)

- 15 (7) A pharmaceutical composition comprising a peptide dimer according to any one of (1) to (6) above together with a pharmaceutically acceptable carrier.

(8) The pharmaceutical composition according to (7) above which is used as a cancer vaccine.

- 20 (9) Use of a peptide dimer according to any one of (1) to (6) above in the manufacture of a cancer vaccine.

(10) A method of treating or preventing cancer, which comprises administering a therapeutically effective amount of a peptide dimer according to any one of (1) to (6) above to a WT1-positive patient in need thereof.

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#### BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 is a graph showing that a peptide dimer (SEQ ID NO: 44) induces CTLs in transgenic mouse.

## BEST MODE FOR CARRYING OUT THE INVENTION

In the peptide dimer of the present invention, two peptide monomers are dimerized through a disulfide bond(s) between SH groups of at least a pair of cysteine residues present in the peptide monomers.

5       The peptide dimer of the present invention has a CTL-inducing activity and the CTLs thus induced can exert an antitumor activity through the cytotoxic effects or the production of lymphokines. Accordingly, the peptide dimer of the present invention can be used as a cancer vaccine for treatment or prevention of cancers (tumors).

10       The peptide monomer constituting the peptide dimer of the present invention consists of 7-30 amino acid residues containing at least one cysteine residue, and produces a tumor antigen peptide having CTL-inducing activity. The phrase "produces a tumor antigen peptide" means that the peptide monomer has a characteristic of rendering a tumor  
15       antigen peptide capable of binding to an HLA antigen and being recognized by cytotoxic T cell (CTL). Any peptide monomer can be used in the present invention without limitation as far as it has a CTL-inducing activity; however, a peptide monomer which is derived from the tumor suppressor gene WT1 of human Wilms' tumor and comprises at least one cysteine  
20       residue is preferred. The tumor suppressor gene WT1 is expressed in various kinds of tumors (Cell, 60:509, 1990; NCBI data base Accession No. XP\_034418, SEQ ID NO: 1). The WT1 gene was isolated from chromosome 11p13 as one of the causative genes of Wilms' tumors based on the analysis of the WAGR syndrome that was complicated by Wilms' tumors,  
25       aniridia, urogenital anomaly, mental retardation, etc. (Nature, 343: 774, 1990). The genomic DNA of WT1 is about 50 kb, and is composed of ten exons, and of which the cDNA is about 3 kb. The amino acid sequence deduced from the cDNA is as shown in SEQ ID NO: 1 (Cell., 60:509, 1990). The WT1 gene has been suggested to promote the growth of leukemia cells  
30       from the facts that the WT1 gene is highly expressed in human leukemia,

and that the leukemia cells are suppressed in their cellular growth by the treatment with WT1 antisense oligomers (JP-A-104627/1997). Then, the WT1 gene has been demonstrated to be a new tumor antigen protein of leukemia and solid cancers (J. Immunol., 164: 1873-80, 2000, and J. Clin. Immunol., 20, 195-202, 2000) from the facts that the WT1 gene is also highly expressed in solid cancers such as gastric cancer, colon cancer, lung cancer, breast cancer, embryonal cancer, skin cancer, bladder cancer, prostate cancer, uterine cancer, cervical cancer, and ovarian cancer (JP-A-104627/1997, WO00/06602). Since cancer immunotherapy (cancer vaccine) is preferably applicable to as many cancer patients as possible, it is significant to identify tumor antigen peptides from WT1 which is highly expressed in many kinds of cancers, and to develop cancer vaccines using the resultant tumor antigen peptides. In this regard, several natural-type tumor antigen peptides consisting of partial fragments of WT1 protein are described in WO00/06602 and WO00/18795; however, nothing has been known about their in vivo effects.

Other peptide monomers usable in the present invention include tumor antigen peptides containing at least one cysteine residue which are derived from tumor antigen proteins listed in the Table of Immunity, vol. 10:281, 1999.

The CTL-inducing activity can be confirmed by measuring the number of CTLs by HLA tetramer method (Int. J. Cancer: 100, 565-570 (2002)) or limiting dilution method (Nat. Med.:4, 321-327 (1998)). Alternatively, for example, in the case of HLA-A24-restricted CTL-induction, the activity can be determined using HLA-A24 model mouse according to the method described in WO02/47474 or Int. J. Cancer: 100, 565-570 (2002).

The peptide monomer consists of 7-30, preferably 8-12, more preferably 9-11 amino acid residues. The peptide monomer preferably contains 1 or 2 cysteine residues taking into account both the motif for

binding with HLA and the length of peptide.

The peptide monomer can be synthesized according to a method generally used in the field of peptide chemistry. Such a method can be found in literatures including Peptide Synthesis, Interscience, New York, 1966; The Proteins, Vol. 2, Academic Press Inc., New York, 1976; Peptide  
5 Synthesis, Maruzen, Inc., 1975; Peptide-Gosei no Kiso to Jikken, Maruzen, Inc., 1985; and Iyakuhin no Kaihatsu (Zoku), Vol. 14, Peptide Synthesis, Hirokawa-syoten, 1991.

The resultant peptide monomers can be allowed to form an  
10 intermolecular disulfide bond according to a method generally used in the peptide chemistry. The method for forming a disulfide bond can be found in literatures including Peptide Synthesis, Interscience, New York, 1966; The Proteins, Vol. 2, Academic Press Inc., New York, 1976; Peptide Synthesis, Maruzen, Inc., 1975; Peptide-Gosei no Kiso to Jikken, Maruzen,  
15 Inc., 1985; and Iyakuhin no Kaihatsu (Zoku), Vol. 14, Peptide Synthesis, Hirokawa-syoten, 1991.

Specifically, a peptide monomer containing one cysteine residue can be synthesized by, for example, removing all the protecting groups including the one on the cysteine side chain, and then subjecting the  
20 resulting monomer solution to air-oxidation under alkali condition, or forming a disulfide bond(s) by adding an oxidizing agent under alkali or acidic condition. Examples of oxidizing agent include iodine, dimethylsulfoxide (DMSO), potassium ferricyanide, and the like.

A monomer peptide containing two or more cysteine residues can be  
25 also synthesized according to the method described above. In this case, isomers resulting from disulfide bonds of different binding manner can be obtained. A peptide dimer wherein a disulfide bond is formed between intended cysteine residues can be prepared by selecting a particular combination of protecting groups for cysteine side chains. Examples of the  
30 combination of protecting groups include MeBzl (methylbenzyl) and Acm

(acetamidemethyl) groups, Trt (trityl) and Acm groups, Npys (3-nitro-2-pyridylthio) and Acm groups, S-Bu-t (S-tert-butyl) and Acm groups, and the like. For example, in the case of a combination of MeBzl and Acm groups, the preparation can be carried out by a method comprising removing protecting groups other than MeBzl group and a protecting group(s) on the cysteine side chain, and subjecting the resulting monomer solution to air-oxidation to form a disulfide bond(s) between the deprotected cysteine residues, followed by deprotection and oxidization with iodine to form a disulfide bond(s) between the cysteine residues previously protected by Acm.

The resultant peptide dimer can be purified according to processes generally used in the field of peptide chemistry. Such a purification method can be found in literatures including Peptide Synthesis, Interscience, New York, 1966; The Proteins, Vol. 2, Academic Press Inc., New York, 1976; Peptide Synthesis, Maruzen, Inc., 1975; Peptide-Gosei no Kiso to Jikken, Maruzen, Inc., 1985; and Iyakuhin no Kaihatsu (Zoku), Vol. 14, Peptide Synthesis, Hirokawa-syoten, 1991. A method using HPLC is preferred.

The resultant peptide dimer of the present invention shows excellent stability against an oxidizing agent or the like in solution and possesses a given quality and CTL-inducing activity due to the disulfide bond(s) between cysteine residues.

Preferred peptide monomers usable in the present invention are illustrated below taking WT1 as an example. As used herein, the following one- or three-letter-abbreviations are used to shorten respective amino acid residues. Ala(A): alanine residue, Arg(R): arginine residue, Asn(N): asparagine residue, Asp(D): aspartic acid residue, Cys(C): cysteine residue, Gln(Q): glutamine residue, Glu(E): glutamic acid residue, Gly(G): glycine residue, His(H): histidine residue, Ile(I): isoleucine residue, Leu(L): leucine residue, Lys(K): lysine residue, Met(M): methionine residue, Phe(F):

phenylalanine residue, Pro(P): proline residue, Ser(S): serine residue, Thr(T): threonine residue, Trp(W): tryptophan residue, Tyr(Y): tyrosine residue, Val(V): valine residue.

In the Table, the term "position" refers to the position of the peptide in human WT1.

Table 1  
HLA-A1-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
137-145	CLESQPAIR	2
80-88	GAEPHEEQC	3
354-362	QCDFKDCER	4
409-417	TSEKPFSCR	5
386-394	KTCQRKFSR	6
325-333	CAYPGCNKR	7
232-240	QLECMTWNQ	8
317-325	TSEKRPFMC	9

Table 2  
HLA-A0201-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
280-288	ILCGAQYRI	10
235-243	CMTWNQMNL	11
227-235	YQMTSQLEC	12
408-416	KTSEKPFSC	13
228-236	QMTSQLECM	14
86-94	EQCLSAFTV	15

Table 3  
HLA-A0205-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
235-243	CMTWNQMNL	11
227-235	YQMTSQLEC	12



194-202	SVPPPVYGC	16
280-288	ILCGAQYRI	10
81-89	AEPHEEQCL	17

Table 4  
HLA-A24-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
356-364	DFKDCERRF	18
326-334	AYPGCNKRY	19
130-138	NAPYLPSC	20
329-337	GCNKRYFKL	21
417-425	RWPSCQKKF	22
207-215	DSCTGSQAL	23
235-243	CMTWNQMNL	11
235*-243	CYTWNQMNL	44

\*: M at position 236 in SEQ ID NO: 11 is altered to Y.

Table 5  
HLA-A3-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
88-96	CLSAFTVHF	24
137-145	CLESQPAIR	2
280-288	ILCGAQYRI	10
386-394	KTCQRKFSR	6
235-243	CMTWNQMNL	11
383-391	FQCKTCQRK	25
194-202	SVPPPVYGC	16

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Table 6  
HLA-A68.1-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
100-108	FTGTAGACR	26

386-394	KTCQRKFSR	6
409-417	TSEKPFSCR	5
325-333	CAYPGCNKR	7
354-362	QCDFKDCER	4
324-332	MCAYPGCNK	27
379-387	GVKPFQCKT	28
137-145	CLESQPAIR	2

Table 7  
HLA-A1101-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
386-394	KTCQRKFSR	6
383-391	FQCKTCQRK	25
100-108	FTGTAGACR	26
324-332	MCAYPGCNK	27
415-423	SCRWPSCQK	29
137-145	CLESQPAIR	2
325-333	CAYPGCNKR	7

Table 8  
HLA-A3101-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
386-394	KTCQRKFSR	6
137-145	CLESQPAIR	2
100-108	FTGTAGACR	26
325-333	CAYPGCNKR	7
279-287	PILCGAQYR	30
354-362	QCDFKDCER	4
383-391	FQCKTCQRK	25
358-366	KDCERRFSR	31

Table 9  
HLA-A3302-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
409-417	TSEKPFSCR	5
137-145	CLESQPAIR	2
354-362	QCDFKDCER	4
100-108	FTGTAGACR	26
325-333	CAYPGCNKR	7
207-215	DSCTGSQAL	23
419-427	PSCQKKFAR	32

Table 10  
HLA-B14-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
329-337	GCNKRYFKL	33

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Table 11  
HLA-B40-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
81-89	AEPHEEQCL	17
410-418	SEKPFSCRW	34
318-326	SEKRPFMCA	35
233-241	LECMTWNQM	36
349-357	GEKPYQCDF	37
85-93	EEQCLSAFT	38
23-31	GCALPVSGA	39
206-214	TDSCCTGSQA	40
24-32	CALPVSGAA	41
84-92	HEEQCLSAF	42

Table 12  
HLA-B60-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
81-89	AEPHEEQCL	17
233-241	LECMTWNQM	36
209-217	CTGSQALL	43
318-326	SEKRPFMCA	35
329-337	GCNKRYFKL	33
130-138	NAPYLPSC	20
85-93	EEQCLSAFT	38
208-216	SCTGSQALL	45
207-215	DSCTGSQAL	23
18-26	LGGGGGCAL	46

Table 13  
HLA-B61-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
318-326	SEKRPFMCA	35
81-89	AEPHEEQCL	17
233-241	LECMTWNQM	36
85-93	EEQCLSAFT	38
206-214	TDCTGSQA	40
20-28	GGGGCALPV	47
23-31	GCALPVSGA	39

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Table 14  
HLA-B62-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
88-96	CLSAFTVHF	24
17-25	SLGGGGGCA	48
384-392	QCKTCQRKF	49
227-235	YQMTSQLEC	12
86-94	EQCLSAFTV	15

101-109	TGTAGACRY	50
280-288	ILCGAQYRI	10

Table 15  
HLA-B7-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
130-138	NAPYLPSCCL	20
208-216	SCTGSQALL	45
18-26	LGGGGGCAL	46
207-215	DSCTGSQAL	23
209-217	CTGSQALLL	43
329-337	GCNKRYFKL	33
235-243	CMTWNQMNL	11

Table 16  
HLA-B8-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
329-337	GCNKRYFKL	33
208-216	SCTGSQALL	45
130-138	NAPYLPSCCL	20
420-428	SCQKKFARS	51
387-395	TCQRKFSRS	52
207-215	DSCTGSQAL	23
384-392	QCKTCQRKF	49
136-144	SCLESQPAI	53
347-355	HTGEKPYQC	54

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Table 17  
HLA-B2702-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
416-424	CRWPSCQKK	55
107-115	CRYGPFGPP	56

Table 18  
HLA-B2705-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
416-424	CRWPSCQKK	55
383-391	FQCKTCQRK	25

Table 19  
HLA-B3501-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
278-286	TPILCGAQY	57
327-335	YPGCNKRYF	58
82-90	EPHEEQCLS	59
207-215	DSCTGSQAL	23
412-420	KPFSCRWPS	60

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Table 20  
HLA-B3701-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
81-89	AEPHEEQCL	17
85-93	EEQCLSAFT	38
208-216	SCTGSQALL	45
209-217	CTGSQALLL	43
206-214	TDCTGSQA	40
84-92	HEEQCLSAF	42
233-241	LECMTWNQM	36
349-357	GEKPYQCDF	37

Table 21  
HLA-B3801-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
202-210	CHTPTDSCT	61
417-425	RWPSCQKKF	22

327-335	YPGCNKRYF	58
208-216	SCTGSQALL	45
18-26	LGGGGGCAL	46
83-91	PHEEQCLSA	62

Table 22  
HLA-B3901-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
136-144	SCLESQPAI	53
208-216	SCTGSQALL	45
207-215	DSCTGSQAL	23

Table 23  
HLA-B3902-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
130-138	NAPYLPSC	20
209-217	CTGSQALLL	43
207-215	DSCTGSQAL	23
208-216	SCTGSQALL	45
329-337	GCNKRYFKL	33

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Table 24  
HLA-B4403-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
349-357	GEKPYQCDF	37
84-92	HEEQCLSAF	42
410-418	SEKPFSCRW	34
278-286	TPILCGAQY	57
318-326	SEKRPFMCA	35
81-89	AEPHEEQCL	17
101-109	TGTAGACRY	50
85-93	EEQCLSAFT	38

233-241	LECMTWNQM	36
104-112	AGACRYGPF	63

Table 25  
HLA-B5101-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
130-138	NAPYLPSCL	20
20-28	GGGGCALPV	47
18-26	LGGGGGCAL	46
418-426	WPSCQKKFA	64
82-90	EPHEEQCLS	59
280-288	ILCGAQYRI	10
204-212	TPTDSCTGS	65

Table 26  
HLA-B5102-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
130-138	NAPYLPSCL	20
20-28	GGGGCALPV	47
412-420	KPFSCRWPS	60
18-26	LGGGGGCAL	46
24-32	CALPVSGAA	66
136-144	SCLESQPAI	53
418-426	WPSCQKKFA	64
351-359	KPYQCDFKD	67



Table 27  
HLA-B5201-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
86-94	EQCLSAFTV	15
20-28	GGGGCALPV	47
327-335	YPGCNKRYF	58
104-112	AGACRYGPF	63

Table 28  
HLA-B5801-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
230-238	TSQLECMTW	68
408-416	KTSEKPFSC	13
276-284	HTTPILCGA	69
347-355	HTGEKPYQC	54
317-325	TSEKRPFMC	9

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Table 29  
HLA-CW0301-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
329-337	GCNKRYFKL	21
24-32	CALPVSGAA	41
136-144	SCLESQPAI	53
130-138	NAPYLPSCL	20

Table 30  
HLA-CW0401-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
356-364	DFKDCERRF	18
327-335	YPGCNKRYF	58
326-334	AYPGCNKRY	19
417-425	RWPSCQKKF	22

278-286	TPILCGAQY	57
99-107	QFTGTAGAC	70

Table 31  
HLA-CW0602-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
130-138	NAPYLPSCl	20
319-327	EKRPFMCAY	71
207-215	DSCTGSQAL	23

Table 32  
HLA-CW0702-restricted Peptide Monomers

Position	Amino acid sequence	SEQ ID NO:
319-327	EKRPFMCAY	71
326-334	AYPGCNKRY	19
278-286	TPILCGAQY	57
327-335	YPGCNKRYF	58
101-109	TGTAGACRY	50
130-138	NAPYLPSCl	20
84-92	HEEQCLSAF	42

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It has been known that there are many subtypes of HLA molecule and that the amino acid sequence of tumor antigen peptide that binds to each subtype obeys a certain rule (binding motif). The binding motif for HLA-A24 is known that, in the peptides consisting of 8 to 11 amino acid residues, the amino acid at position 2 is tyrosine (Tyr), phenylalanine (Phe), methionine (Met) or tryptophan (Trp), and the amino acid at the C-terminus is phenylalanine (Phe), leucine (Leu), isoleucine (Ile), tryptophan (Trp) or methionine (Met) (J. Immunol., 152, p3913, 1994; Immunogenetics, 41, p178, 1995; J. Immunol., 155, p4307, 1994). Accordingly, in addition to the peptide monomers in Table 4, a peptide monomer of the following

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formula can also be preferably used as an HLA-24-restricted peptide monomer.

Cys Xaa Thr Trp Asn Gln Met Asn Xaa (SEQ ID NO: 72)

wherein Xaa at position 2 is an amino acid residue selected from Tyr, Phe, Met and Trp; and Xaa at position 9 is an amino acid residue selected from Phe, Leu, Ile, Trp and Met.

The binding motif for HLA-A0201 is known that, in the peptides consisting of 8 to 11 amino acid residues, the amino acid at position 2 is leucine (Leu) or methionine (Met), and the amino acid at the C-terminus is valine (Val) or leucine (Leu). The binding motif for HLA-A0205 is known that, in the peptides consisting of 8 to 11 amino acid residues, the amino acid at position 2 is valine (Val), leucine (Leu), isoleucine(Ile) or methionine (Met) and the amino acid at the C terminus is leucine (Leu) (Immunogenetics, 41, p.178, 1995; J. Immunol., 155: p.4749, 1995). Accordingly, a peptide wherein the amino acid at position 2 or the C terminus of a peptide monomer shown in Table 2 or 3 above is substituted by any one of amino acid motifs described above can also be preferably used as an HLA-A0201- or HLA-A0205-restricted peptide monomer.

The peptide monomers shown in Table 4 above are especially preferred to be used in the present invention. Among the peptides in Table 4, the SEQ ID NO:44 is a non-natural variant peptide wherein the methionine at position 236 of SEQ ID NO: 11 (position 235-243) is altered to tyrosine. Accordingly, the peptide monomers of the present invention include those having a sequence wherein one or more amino acid residues other than cysteine residue are altered in the sequence of natural-type peptides and showing CTL inducing activity.

As another embodiment, the present invention provides a pharmaceutical composition comprising a peptide dimer of the present invention together with a therapeutically acceptable carrier therefor. Although the amount of a peptide dimer of the present invention as an

active ingredient in the pharmaceutical composition may vary depending on the purpose of treatment, the age, weight of the patient, and the like, it is typically 0.0001mg to 1000mg, preferably 0.001mg to 1000 mg, more preferably 0.1mg to 20mg.

5           The pharmaceutical composition of the present invention may comprise, as an active ingredient, a peptide monomer in addition to a peptide dimer of the present invention. There is no limitation about the content of a "peptide dimer" in the pharmaceutical composition of the present invention on the condition that the CTL inducing activity is  
10           exerted; however, it can be 50% or more, preferably 70-100%, and more preferably 80-100% of the whole peptides. The content of a peptide dimer can be confirmed by high performance liquid chromatography (HPLC).

          The pharmaceutically acceptable carriers are those being capable of enhancing the cellular immunity. Such carriers include an adjuvant.  
15           Examples of adjuvant applicable to the present invention include those described in a literature (Clin. Microbiol. Rev., 7: 277-289, 1994), specifically, components derived from microorganisms, cytokines, components derived from plants, mineral gels such as aluminium hydroxide, lysolecithin, surfactants such as Pluronic® polyols, polyanion,  
20           peptide, oil emulsion (emulsion preparation) and the like. Also, the carrier includes components required for the preparation of liposomal preparations, particulate preparations in which the ingredient is bound to beads having a diameter of several  $\mu\text{m}$ , preparations in which the ingredient is attached to lipids, and the like.

25           Administration may be achieved, for example, intradermally, subcutaneously, intramuscularly, or intravenously. Preferred route is intradermal or subcutaneous administration that induces CTLs efficiently. The frequency or interval of administration can be adjusted appropriately depending on the disease to be treated or prevented, and individual  
30           difference; however, the administration is preferably carried out more than

one times at an interval of once in a several days to several months.

For example, when the pharmaceutical composition of the present invention comprising a peptide dimer consisting of peptide monomers derived from WT1 is administered to a WT1-positive patient, the peptide is presented to an HLA antigen of antigen-presenting cells to form a complex. CTLs specific for the presented HLA antigen complex are then proliferated and destroy cancer cells, whereby cancer can be treated or prevented. The pharmaceutical composition of the present invention can be used to treat or prevent cancers associated by the elevated expression level of WT1 gene including blood cancers such as leukemia, myelodysplastic syndrome, multiple myeloma and malignant lymphoma, and solid cancers such as gastric cancer, colon cancer, lung cancer, breast cancer, embryonal cancer, hepatic cancer, skin cancer, bladder cancer, prostate cancer, uterine cancer, cervical cancer, and ovarian cancer.

In the further embodiment, the present invention provides a method for treating or preventing cancers by administering the pharmaceutical composition of the present invention to a WT1-positive patient.

## EXAMPLES

The present invention is further illustrated by the following examples, but is not limited by these examples in any respect.

### PREPARATION 1

#### 1. Synthesis of Protected Peptide Resin (H-Cys(Trt)-Tyr(Trt)-Thr(tBu)-Trp(Boc)-Asn(Trt)-Gln(Trt)-Met-Asn(Trt)-Leu-Alko-Resin)

Fmoc-Leu-Alko-resin (wherein Alko is p-alkoxybenzyl alcohol) (12 g) (0.81 mmol/g, Watanabe Chemical Industries, Ltd.) was charged in a reaction vessel (500 ml, Type ACT90 solid phase synthesizer, Advanced ChemTech) and washed once with DMF or the like (Process 1). The resin was then treated with 25% Pip (piperidine) (3 minutes × 1, and 15 minutes × 1) to cleave the Fmoc group (Process 2), and washed again with DMF or

the like (Process 1 ) to remove Pip. To the reaction vessel was added a solution of Fmoc-Asn(Trt)-OH (29.36 g) and HOBT (1-hydroxybenzotriazole) (7.5 g) in NMP (N-methylpyrrolidinone) (150 ml). After adding DIPCI (N,N'-diisopropylcarbodiimide) (7.6 ml), the mixture was stirred at room temperature for 30 minutes (Process 3). Thirty minutes later, the resin was washed with NMP (Process 4), and subjected to the coupling reaction once again using Fmoc-Asn(Trt)-OH (29.36 g) and HOBT (7.5 g) (Process 5) to synthesize Fmoc-Asn(Trt)-Leu-Alko resin. The resultant resin was then converted to H-Asn(Trt)-Leu-Alko-resin by repeating the deprotection of Process 2. After washing (Process 1), Fmoc-Met-OH (18.27 g), Fmoc-Gln(Trt)-OH (30.04 g), Fmoc-Asn(Trt)-OH (29.36 g), Fmoc-Trp(Boc)-OH (25.91 g), Fmoc-Thr(tBu)-OH (19.56 g), Fmoc-Tyr(tBu)-OH (22.60 g) and Fmoc-Cys(Trt)-OH (28.82 g) were added in series to conduct the coupling reaction (Process 3), wherein the coupling was repeated three times with Fmoc-Thr(tBu)-OH. The resultant resin was washed with DMF and treated with 25 % AC<sub>2</sub>O (acetic anhydride) (15 minutes × 2) for the capping of unreacted amino groups. Following condensation of the N-terminal Fmoc-Cys(Trt)-OH, the deprotection (Process 2) and washing (Process 6) were conducted to obtain H-Cys(Trt)-Tyr(Trt)-Thr(tBu)-Trp(Boc)-Asn(Trt)-Gln(Trt)-Met-Asn(Trt)-Leu-Alko-Resin. The above processes for synthesis are summarized in Table 33.

Table 33

<Processes for Synthesis>			
Process	Reagent	Number of treatment	Time (min)
1) Washing	DMF	100ml ×6	0.3
	MeOH	100ml ×1	0.3
	DMF	100ml ×3	0.3
2) Deprotection	25% piperidine/DMF	100ml	3.0
		100ml	15.0
3) Coupling	Amino-protected amino acid (5 eq. for each), HOBT (5 eq.), DIPCI (5 eq.)/NMP	150ml	30×1
4) Washing	NMP	100ml×2	0.3
5) Coupling	Amino-protected amino acid (5 eq. for each), HOBT (5 eq.), DIPCI (5 eq.)/NMP	150ml	30×1
6) Washing	DMF	100ml ×5	0.3
	MeOH	100ml ×1	0.3
	DMF	100ml ×2	0.3

## 5      2.      Deprotection of Protected Peptide Resin

To the protected peptide resin (H-Cys(Trt)-Tyr(Trt)-Thr(tBu)-Trp(Boc)-Asn(Trt)-Gln(Trt)-Met-Asn(Trt)-Leu-Alko-Resin) (14.06 g) obtained in accordance with the processes above were added Reagent K (5% phenol/5% thioanisole/5% H<sub>2</sub>O/2.5% ethanediol/TFA solution, 100 ml) and triisopropylsilane (TIPS, 15 ml), and the mixture was stirred at room temperature for 2.5 hours. After adding diethyl ether (ca. 500 ml), the mixture was filtered through a glass filter to remove Reagent K and diethyl ether as filtrate. The residue on the filter was washed with diethyl ether (ca. 100 ml, x3) followed by addition of TFA (ca. 100 ml x 3) to obtain filtrate (300 ml) containing the objective product. The filtrate was concentrated to remove TFA and lyophilized after adding acetonitrile (ca.

50 ml) and 20% aqueous acetic acid solution (ca. 250 ml) to obtain a crude peptide (H-Cys-Tyr-Thr-Trp-Asn-Gln-Met-Asn-Leu-OH, SEQ ID NO:44) (6.12 g) as powder.

### 3. Purification of Crude Peptide

5           The resultant crude peptide (749 mg) was dissolved in TFA (10 ml) and charged onto ODS C<sub>18</sub> column (5 cm  $\Phi$   $\times$  50 cm L, YMC, Co., Ltd.) of HPLC (Shimadzu; LC8AD type) equilibrated with solution 1 (= H<sub>2</sub>O/0.1% TFA) using an HPLC pump. The column was kept for about 30 minutes as it is, and then the concentration of solution 2 (= CH<sub>3</sub>CN/0.1% TFA) was  
10 increased from 0% to 15% over 30 minutes. Thereafter, the concentration of solution 2 was increased upto 28 % over 330 minutes, while the eluate containing the objective peptide was monitored by UV absorption at 220 nm to collect the fractions containing the objective product. The fractions were combined and injected into ODS C<sub>18</sub> column (4.6 mm  $\Phi$   $\times$  25 cm L, YMC, Co., Ltd.) attached to HPLC (Hitachi, L-4000 type) and equilibrated  
15 with 17% solution 2 (= CH<sub>3</sub>CN/0.1% TFA) in a mixture of solution 1 (= H<sub>2</sub>O/0.1% TFA) and solution 2 (= CH<sub>3</sub>CN/0.1% TFA), and then the concentration of solution 2 was increased upto 47% over 30 minutes while monitoring the eluate by UV absorption at 220 nm over 30 minutes to  
20 obtain the purified objective peptide monomer (227.5 mg) with retention time of 14.79 minutes.

### Amino acid analysis

25           Hydrolysis: 1% phenol/6N aqueous hydrochloric acid solution  
110 °C, 10 hours  
Analytical method: ninhydrin method  
Asx:1.71(2) Thr:0.75(1) Glx:1.07(1) Met:0.91(1) \* Leu:(1)  
Tyr:0.82(1)  
\*) Leu = reference amino acid  
30           The value in parentheses ( ): theoretical value



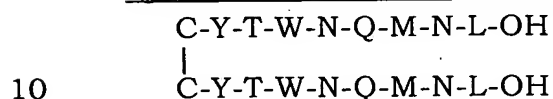
Mass spectrometry: LC/MS  $M^{+1} = 1173.0$  (theoretical value = 1172.36)

Peptide Sequencing: sequence was confirmed from the second residue (Tyr) from the N-terminus to the C-terminus, Leu, successively.

5

#### EXAMPLE 1

##### Synthesis of a Dimer of the Formula:



Air oxidization was conducted by stirring a mixture of a peptide monomer (227.5 mg) prepared in Preparation 1, N-methylglucamine (NMG) (227.5 mg) and water (23 ml) at room temperature for about 2 days. To the  
15 reaction solution was added an aqueous solution of sodium acetate (2 g) in water (5 ml), and the mixture was stirred at room temperature for about 20 minutes. After adding water (200 ml) and acetonitrile (ca. 200 ml), the mixture was filtered through Kiriyaama Roht (filter paper No. 5C), and the residue on the filter was washed with water (ca. 50 ml  $\times$  3). The residue on  
20 the filter was collected and lyophilized after adding water (ca. 200 ml) to obtain the crude product of objective peptide dimer (158 mg).

##### Purification of Crude Peptide Dimer

Crude peptide dimer (158 mg) was dissolved in DMSO (9 ml) and charged onto ODS  $C_{18}$  column (5 cm  $\Phi$   $\times$  50 cm L, YMC, Co., Ltd.) of HPLC  
25 (Shimadzu; LC8AD type) equilibrated with solution 1 (=  $H_2O$ /1% AcOH) using a HPLC pump. The column was kept for about 30 minutes as it is, and then the concentration of solution 2 (=  $CH_3CN$ /1% AcOH) was increased from 0% to 40% over 360 minutes. Thereafter, the fractions containing the objective product were collected by means of automatic  
30 fraction collector while monitoring the eluate containing the objective peptide dimer by UV absorption at 220 nm. The fractions were combined

and injected into ODS C<sub>18</sub> column (4.6 mm  $\Phi$   $\times$  25 cm L, YMC, Co., Ltd.) attached to HPLC (Hitachi, L-4000 type) and equilibrated with 17% solution 2 (= CH<sub>3</sub> CN/0.1% TFA) in a mixture of solution 1 (= H<sub>2</sub> O/0.1% TFA) and solution 2 (= CH<sub>3</sub> CN/0.1% TFA). The concentration of solution 2 was then increased from 0% to 47% while monitoring the eluate by UV absorption at 220 nm over 30 minutes to obtain the purified objective peptide dimer (46.6 mg) with retention time of 20.51 minutes. FAB.MS 2365.0 (theoretical value: 2342.70) Na<sup>+</sup> F = 0.25%

## 10 TEST EXAMPLE 1

### Induction of CTLs with Peptide Dimer

The CTL-inducing activity of the peptide dimer prepared in Example 1 was evaluated using HLA-A24 transgenic mice (Int. J. Cancer: 100, 565, 2002). The peptide dimer was dissolved in dimethyl sulfoxide (DMSO) to obtain a 40mg/ml peptide solution. The peptide solution (35  $\mu$ l) was then added to 10 mM phosphate buffer (pH 7.5) (581  $\mu$ l) to obtain a peptide suspension. The resultant peptide suspension (550  $\mu$ l) and Montanide ISA51 (Seppic) (700  $\mu$ l) were mixed using a connected glass syringe to prepare an emulsion as an administration solution.

The administration solution (200  $\mu$ l) was injected into an HLA-A24 transgenic mouse subcutaneously in the base of the tail. Three mice were used. Seven days after the injection, the spleen was removed and splenocytes were prepared. A portion of the splenocytes was pulsed with the peptide dimer (100  $\mu$ g/ml) for 1 hour. Splenocytes not pulsed with the peptide were seeded into a 24-well plate at  $7 \times 10^6$  cells/well and thereto were added the above-mentioned splenocytes pulsed with the peptide ( $1 \times 10^6$  cells/well), and the plate was incubated. The incubation was conducted in RPMI1640 medium supplemented with 10% FCS, 10 mM HEPES, 20 mM L-glutamine, 1 mM sodium pyruvate, 1 mM MEM nonessential amino acids, 1% MEM vitamin and 55  $\mu$  M 2-

mercaptoethanol for 5 days.

The cultured splenocytes were examined for the cytotoxic activity specific for the peptide used in the administration by  $^{51}\text{Cr}$  release assay (J. Immunol.: 159, 4753, 1997). EL4-A2402/K<sup>b</sup> cells obtained by transforming EL-4 cells (ATCC No. TIB-39) in such a manner that a chimera MHC class I molecule of HLA-A24 and H2K<sup>b</sup> (Int. J. Cancer: 100, 565, 20002) are expressed stably were used as the target cells. The target cells were labeled with  $^{51}\text{Cr}$  (3.7 MBq/ $10^6$  cells) and pulsed with the peptide at 100  $\mu\text{g}/\text{ml}$  for an hour. For control, target cells not pulsed with the peptide were labeled with  $^{51}\text{Cr}$  for 2 hours. Those labeled target cells and the previously prepared splenocytes were mixed at a ratio of 1:120, cultured for 4 hours and the CTL activity was evaluated on the basis of the percent of damaged target cells. The results are shown in Fig. 1. The splenocytes prepared from the mouse injected with the peptide injured strongly the target cells pulsed with the peptide. However, they showed only weak cytotoxicity on the target cells not pulsed with the peptide. These results clearly showed that CTLs specific for the peptide were induced.

## INDUSTRIAL APPLICABILITY

According to the present invention, a peptide dimer having a CTL-inducing activity in vivo, and pharmaceutical compositions comprising the same as an active ingredient are provided. The present invention can be useful in the improvement of conditions of many tumor patients.